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Supplementary Materials for

Therapeutic responses to *Roseomonas mucosa* in atopic dermatitis may involve lipid-mediated TNF-related epithelial repair

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at stm.sciencemag.org/cgi/content/full/12/560/eaaz8631/DC1)

Data file S1 (Microsoft Excel format). MetaboAnalyst report file for metabolomics assessments.
Date file S2 (Microsoft Excel format). Individual-level data for all figures.

Supplementary Materials

Materials and Methods

Patient recruitment
Patients were recruited and enrolled at the NIH under clinical trial NCT03018275 (Beginning Assessment of Cutaneous Treatment Efficacy of *Roseomonas* in Atopic Dermatitis, Phase I/II; BACTE*R*iAD I/II). Two investigators documented the surface areas involved and intensity of disease. One investigator was intentionally misinformed that the study was a placebo-controlled design and therefore was unaware that all patients were on active treatment with the investigational drug. Only scores from the blinded investigator are shown. Patients provided the subjective values for pruritus and sleep disturbance.

Screening blood work included complete blood count, chemistry panel, calcium, magnesium, phosphorus, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, bilirubin, and albumin. There were no restrictions on home medication use except for those specified in the exclusion criteria. Pediatric patients were asked to maintain their standard approach; parents reported using daily emollients but reserving steroid treatments for flares. Parents were asked to recall average days/month of topical steroid use for the 3 months prior to treatment and then prospectively track steroid use during treatment. Transepidermal water loss was measured by VapoMeter (Delfin) per the manufacturer's instructions and represents the average results of the bilateral antecubital fossae.

Sample size derivations
Sample size demonstrated that with 15 total patients, there would be a probability of 0.14 of observing 1 or more serious adverse events (SAEs) or AEs (grade 2 or higher as defined by the DAIDS toxicity table) if the true rate were 0.01, and a probability of 0.8 of observing 1 or higher if the true rate were 0.1 by binomial distribution. An independent safety monitor assessed all clinical and laboratory data. Activity sample size calculations were based on a 50% reduction in SCORAD compared to the historical placebo response and natural history of self-resolution (2). With 15 total participants, if 4 successes were observed at the end of the study then there would be a 0.9 probability (90% power) of concluding the treatment were active if the true activity rate were 0.4. These calculations were based on the binomial distribution.

Endpoints
Primary endpoints were (a) frequency of solicited and unsolicited adverse events, serious adverse events, and death; and (b) 50% reduction in SCORAD. Secondary endpoints were (a) a 50% reduction in EASI, (b) 30% improvement in the Children's Dermatology Life Quality Index (CDLQI); and (c) 30% improvement in the Family Dermatology Life Quality Index (FDLQI).

Inclusion criteria
Inclusion criteria were (a) age 18 years or older (adult cohort) or age 7–17 (pediatric cohort); (b) SCORAD of at least 10; (c) carry a physician diagnosis of AD with active involvement of the antecubital fossa; (d) willing to allow storage of blood for future research; (e) no history of other skin disease; (f) initiated or attempted standard of care therapy at least 6 months prior to enrollment; and (g) agreement to use adequate contraception if indicated.

Exclusion criteria
Exclusion criteria were (a) presence of an indwelling venous or arterial catheter; (b) individuals living with anyone with a diagnosed immunodeficiency, cardiac valvular disease, and/or indwelling catheter; (c) presence of allergies to amikacin, ciprofloxacin, gentamicin, levofloxacin, and tobramycin (which would preclude treatment of any unexpected infection); (d) history of cardiac valvular disease; (e) any history of grade 2 or higher neutropenia or leukopenia; (f) clinical suspicion of immunodeficiency, liver disorder,

kidney disorder, and/or HIV; (g) pregnant or breastfeeding; (h) any history of anti–tumor necrosis factor (TNF) treatment; (i) inability to demonstrate proper bacteria administration procedure despite coaching and training; (j) use of any antibiotics within 4 weeks of enrollment; (k) use of oral steroids within 4 weeks of enrollment; and (l) any condition that, in the opinion of the investigator, contraindicates participation in this study.

R. mucosa pharmaceutical formulation

Preparations of *R. mucosa* for clinical use were produced under investigational new drug (IND) application 17303 from the Food and Drug Administration (FDA). Three isolates of *R. mucosa* taken from 3 HVs were grown in minimal media (R2A broth, Teknova) for 24–48 hours. The bacterial cells were washed 3 times in PBS (Gibco) and resuspended into 10%–15% sucrose in water for a concentration of 10^9 CFU/ml based on previously reported growth parameters (*2*). Serial dilutions were performed in 10%–15% sucrose to generate stocks of 10^4, 10^5, and 10^6 per ml. Aliquots of diluted bacterial samples were plated on R2A agar (Remel; San Diego, CA) and incubated at 32°C for 48–72 hours to enumerate pre-lyophilization CFU concentration. Starting CFU values were 90%–105% of expected concentrations. 1.5 ml of bacterial solution was frozen in 3-ml self-contained sprayer system (Discount Vials; Madison, WI) prior to lyophilization (Labconco; Kansas City, MO). Vials/sprayers were sealed, labeled, and stored at –70°C until dispensed to the patients. Three aliquots per batch were reconstituted in sterile water and plated after serial dilution to enumerate post-lyophilization CFU concentration. Survival after lyophilization was 93%–99% of starting CFU. These aliquots were also plated on soybean-casein digest agar (BD Bioscience; San Jose, CA), Sabouraud dextrose agar (Remel), MacConkey agar (BD Bioscience), xylose lysine agar (Remel), charcoal agar (BD Bioscience), and mannitol salt agar (Remel) and assessed for the presence of contaminating bacteria as per FDA *USP 61/62*. No contamination was found in any batches of *Roseomonas* treatment. To avoid batch effects, all doses were derived and vialed in one session.

R. mucosa dosing and application

We have previously reported results from ten adults and five pediatric patients (*2*). In this study, fifteen additional pediatric patients were enrolled and provided bacteria lyophilized in a self-contained sprayer system (Discount Vials). Eyedroppers (United States Plastic Corp; Lima, OH) of 1.5 ml sterile water were provided. For each dose, patients or their parents were instructed to empty the contents of the eyedropper into the sprayer vial, wait 2–5 minutes for reconstitution, and then spray. Sprayers were metered so that 3 pump sprays mirrored the 250µl applied in the adult trial. Dose concentrations of CFU/ml were identical to the previously described (*2*). Pediatric patients applied the treatment twice weekly for the first 3 months of treatment, then every other day for the final month. Dose escalations were every 4 weeks after safety assessments. After completion of dose escalation in 15 total patients, subsequent enrollees were initiated on treatment at 10^5 twice weekly for 12 weeks, then escalated to every-other-day for the final 4 weeks. Treatments were paused during the one-week of active HFM disease in patient 2.19. Patient 2.16 missed one week of the 10^3 therapy while on a road trip with friends. No other missed doses were reported by any patient.

Skin Swab and microbial DNA extraction

FloqSwabs (519CS01; Copan; Murrieta, CA) pre-moistened with phosphate buffer solution (Corning; Corning, NY) before rubbing on one square inch of the patients' skin. *S. aureus* and CNS burden was determined by vortexing swabs in 2 ml of typsin broth (Remel) for 30 seconds and plating 100 µl on blood agar plates (Remel). The following day, the number of colonies was enumerated and multiplied by 20 to obtain the total CFU in the 2-ml collection volume and then averaging values between both arms. Relative abundance of *S. aureus* was obtained by dividing the colony numbers for *S. aureus* by those for coagulase negative *Staphylococci*.

For patients 2.06-2.21, DNA from skin swabs were extracted from swabs intended that were frozen until patch extraction under a bio-safety cabinet. "Air swabs" were used as negative batch controls. 150mcL of Yeast Cell lysate buffer (Lucigen MPY80200; Middleton, WI) was added to the swab tube and another 150mcL was added to a new 1.5mL sterile tube (Eppendorf; Hauppauge, NY). Swabs and any remaining fluid were placed in a filter basket (DNA IQ; Promega; Madison, WI) and centrifuged at 13000 rpm (Eppendorf 5417R) for 30sec at 4^{O}C. The basket and swab were discarded and 0.2mL of ready-lyse lysozyme (Lucigen) was added to each tube. After vortexing on low for 5sec tubes sat at room temperature for 30 minutes. 0.1mm zirconia/silica microbeads (BioSpec; Bartlesville, OK) were added to a new screw-top tubes (Eppendorf) approximately the level needed to fill the conical end. The 300mcL of volume was moved from the incubating tubes into the new tubes with the beads. Tubes were shaken in a homogenizer (Fisher brand Bead Mill 4) at 5m/s for 40 seconds. They were then centrifuged as above for 15secs and the supernatant moved to another, new 1.5mL tube. 5mcL of proteinase K (Thermo) was added to each and they were incubated at 56^{O}C for 30min at 900rpm on an Eppendorf thermomixer C. After incubation, tubes were centrifuged at 13000rpm for 10sec at 4^{O}C, then placed on ice for 5min. 150mcL of MPC protein precipitation was added and the tubes were vortexed on low for 10sec. Samples were then centrifuged at 14800rpm for 10min at 4^{O}C. Supernatants were transferred to a final 1.5mL tube and the pellet discarded. 500mcL of ice-cold isopropanol was added and the tubes inverted 35 times. Samples were then centrifuged at 13000rpm for 10min at 4^{O}C. Supernatants were discarded without disturbing the resulting pellet. 500mcL of 70% ice-cold ethanol was added, then samples were centrifuged at 13000rpm for 1min at 4^{O}C. Again, supernatants were discarded without disturbing the resulting pellet, a new allotment of 500mcL of 70% ice-cold ethanol was added, then samples were centrifuged at 13000rpm for 1min at 4^{O}C. All supernatant was carefully removed, and the pellet left to air dry for 10min at room temp. 35mcL of sterile water was added and samples were frozen at -70^{O}C until qPCR was performed.

Cell culture stimulation and antibody modulation

All bacterial stimulations were performed at MOI of 1. Antibody blockade was performed using a final concentration of 1ug/mL of anti-TNFR1 (Thermo Fisher Scientific; Waltham, MA; H398), TNFR2 (Thermo; 22221.311), CXCR2 (R&D; Minneapolis, MN; 48311), c-Met (Thermo; PA1-28026), TLR5 (InvivoGen; San Diego, CA; Pab-hTLR5), TLR4/MD2 (InvivoGen; Mab-hTLR4/MD2), TLR2 (InvivoGen; TL2.1); an equal combination of TGFβ1 (Thermo; 234R32), TGFβ2 (Thermo; OTI3B4), and TGFβ3 (Thermo; MA5-17187); or isotype controls for mouse IgG1κ, IgG2a, IgG2b (Thermo); goat IgG (Thermo); or rat IgG (InvivoGen). Mecamylamine and atropine (Sigma Aldrich; St. Louis, MO) were used at a final concentration of 5nM. Carbachol (Sigma Aldrich) was used at a final concentration of 1μM. Ketanserin (Sigma) was used at a final concentration of 10nM. Lipid or aqueous fraction stimulation were performed at a final concentration of 2% v/v. Cell supernatant cytokines were collected at 24 hours for all cell types and assessed by multiplex analysis per manufacturer instructions (BioRAD). Aqueous and lipid separation was performed on 10^{7} CFU of bacteria using the Bligh-Dyer method and dried under nitrogen. Lipid fractions were resuspended to stock concentrations of 10^{8} CFU equivalents in 100% ethanol prior to use.

Human cell RNAseq

RNAseq libraries were created in triplicate for all cell types using Illumina TruSeq Stranded Total RNA Library Prep, pooled, and sequenced on a HiSeq4000 producing paired-end 150 base-pair reads. RNA-seq data was processed using the Pipeliner workflow (https://github.com/CCBR/Pipeliner). Reads were trimmed using cutadapt (*76*) and aligned to the human hg38 reference genome and Gencode release28 using STAR v2.5.2b (*73*). RSEM v1.3.0 (*80*) was used for gene-level expression quantification, and limma v 3.40.2 (*77*) was used for *voom* quantile normalization and differential expression analysis. An adjusted p-value (false discovery rate, FDR) of 0.05 was considered significant. Only genes with more than 0.5 counts per million across at least three samples were used for downstream analyses. Pathway

analysis was accomplished using Ingenuity Pathway Analysis (Qiagen). The accession numbers for the RNA-seq dataset is NCBI BioProject: GSE146184.

Bacterial transcriptomics

Roseomonas mucosa freezer stocks were streaked onto R2A (Teknova, Hollister, CA) agar plates and incubated for 72 hrs at 32^{O}C. Single colonies were chosen to inoculate 8 ml of R2A broth in a 15 ml snap-cap Falcon tube and incubated on a slant at 32^{O}C with 250 rpm. After 17 hrs, 3 ml of culture was used to inoculate 50 ml of pre-warmed R2A broth and grown to mid-exponential phase at 32^{O}C with 150 rpm. Upon reaching an OD600 of 0.4, 10 ml of culture was combined with 20 ml of RNAprotect (Qiagen, Valencia, CA), incubated at room temperature for 5 min and centrifuged at 5000 x g for 10 min to pellet bacteria. Upon careful removal of the supernatant, the bacterial pellet was frozen at -80^{O}C. Nucleic acids were extracted from cell pellets using the ALL Prep kit (Qiagen; Hilden, Germany) with on-column DNase treatment following manufacturer's directions. Ribosomal RNA was depleted from the samples using Bacterial RiboZero (Illumina; San Diego, CA) according to manufacturer's instructions. Sequencing libraries were generated using the TruSeq Stranded Total RNA sample prep kit (Illumina) following manufacturer's instructions. Barcoded libraries were sequenced on a NextSeq550 (Illumina) using 150 cycle, mid-output sequencing chemistry, configured for 2 x 75 bp paired end reads, and generated ~14 million reads per sample.

Sequencing reads were trimmed of adapter sequences using CutAdapt version 1.12 (*76*) and trimmed and filtered for low quality bases and reads using the FASTX-Toolkit (Hannon Lab, CSHL, NY). Resulting reads were aligned to the *Roseomonas mucosa* AD1 genome using Bowtie 2 version 2.2.0 (*74*). Reads were assigned to transcripts using htseq-count (*72*) and differential gene expression was performed using DESeq2 (*75*) comparing the 3 Healthy Volunteer isolates to the 3 Patient derived isolates. Since the plasmid content of the 6 strains varied greatly, DESeq2 analysis was performed after removal of all plasmid genes. DESeq2 analysis was also performed after removal of isolate HV3.

Strain-specific qPCR

Genome sequences of *Rm*HV1-3 were compared. Unique sequences were identified for each strain, a hypothetical protein RKNM01394, N-6 DNA methylase RHFA01191, and N-6 DNA methylase RSQQ03345 contained unique sequences. Q-RT-PCR probe and primer sets were designed using Primer Express version 3.0 (Life Technologies, Carlsbad, CA; Table S5)

Forward and reverse primers were designed to be compatible in a multiplex reaction. The fluorescent dyes were selected, which had different emission spectra and also can be detected in ABI 7500 Real-time PCR system. At RTS/Genomics we used ABI QuantStudio 12K Flex Real-Time PCR System to perform the testing. Once we had designed primers and probes, we used BLAST to determine the specificity of the designs. Each design amplicon was compared with other sequenced *Roseomonas* species and other bacteria. *Rm*HV2 strain amplicon showed no significant hits to other known *Roseomonas* strains, whereas *Rm*HV3 strain amplicon showed high homology to 5 additional *Roseomonas spp*. Similarly, *Rm*HV1 strain amplicon showed homology to additional *Roseomonas spp.* QPCR oligos were ordered from LCG Biosearch Technologies. Genomic DNA was extracted from *in vitro* cultured *Rm*HV1-3 stains using Qiagen AllPrep DNA/RNA mini kit following manufacturer's recommendations (Valencia, CA). The Invitrogen Express QPCR Supermix Universal with premixed ROX (Life Technologies) was used to perform the assay. The reactions were carried out in 20 µL reactions (1X), forward primer (0.4µM), reverse primer (0.4µM), and fluorescent probe (0.120µM). Standard curve was prepared from each bacterial strain gDNA. gDNA was sequentially diluted eight times at 1:5 ratio starting with 2ng/µL dilution. The QPCR reactions were carried out at 50 °C for 2 minutes, 95 °C for 2 minutes, 55 cycles of 95 °C for 15 seconds, and 60 °C for 1 minute. Data was analyzed using ABI 7500 Fast version 2.4 sequence detection system software (Life Technologies). CFU equivalents were calculated by generating cycle threshold-to-CFU curves from DNA extracted from enumerated *Rm*HV cultures. Comparisons

were made to cycle threshold values for each isolate signal from a given skin swab. Neither the air swab negative controls nor the day 0 swabs demonstrated qPCR signal.

Acetyl choline and phosphatidylcholine detection assays
Enzymatic assays for total phospholipid, phosphatidylcholine, and acetylcholine were performed using commercial kits from Cell Biolabs, Inc (San Diego, CA) following manufacturer instructions. Phospholipid fluorometric analysis was performed on BioTek Synergy H1 (Winooski, VT). Acetylcholine analysis by colormetric was performed on BioRad Benchmark Plus (Hercules, CA). Cultures were grown overnight; an aliquot was taken from each for serial dilution and plating on R2A plates as described above. Bacteria were then pelleted, rinse once with PBS and then processed per instructions. After CFU concentrations were enumerated, assay values were adjusted per CFU for each individual isolate.

Motility agar assay
Motility agar was derived by mixing 125mg each for: peptone (LP0085; Oxoid, Hampshire, England), casein hydrolysate (Sigma Aldrich), starch (Sigma Aldrich), yeast extract (Fischer Scientific; Fair Lawn, ND); 75mg for K_2HPO_4 (USP; Rockville, MD) and sodium pyruvate (Sigma Aldrich); 12.5mg $MgSO_4$ (Sigma Aldrich); and 1g agar (Life Technologies; Carlsbad, CA) in 250mL of water. Wells of a 12 well plate were filled halfway and the agar allowed to set overnight at $4^{O}C$. One bacterial loop (BD Bioscience; Sparks, MD) full was inserted vertically into the agar and removed. Loops without bacteria were used to assess for contamination as well as angle of insertion. After 48 hours, images were taken of the bacterial isolates and area of growth calculated using IMARIS as in the scratch assay.

Histology
Tissues were processed, sectioned, and stained with hematoxylin and eosin (H&E) by Histoserv (Germantown, MD). Tissues were evaluated by a board-certified veterinary pathologist and photomicrographs were taken using a Leica DMI6000B microscope camera (Wetzlar, Germany).

Full-thickness skin equivalent TEER
Neonatal human dermal fibroblasts (NDFs) were purchased from ATCC (ATCC, PCS-201-010, Manassas, VA, USA). Neonatal human epidermal keratinocytes (NHEKs) were purchased from Sciencell (Sciencell, 2100, Carlsbad, CA, USA). Fibroblast were cultured in MEM medium supplemented with 5% FBS (Thermo Fisher Scientific, Waltham, MA, USA). NHEKs were cultured in keratinocyte growth medium (KGM) purchased from Sciencell (Sciencell, Carlsbad, CA, USA). All the cells were maintained at $37^{o}C$ under a 5% CO_2 atmosphere. Cells of passage number 3-4 were used.

For customized transwell bioprinting, the HTS 96 well transwell (Corning 3377, Corning, NY, US) was glued (Kwik-cast sealant, world precision instrument, Sarasota, FL, USA) with an electrospun scaffold. The scaffold was made of PLGA nanofibers with 900 nm diameter size and 4 μm thickness (BioSurfaces, Ashland, MA). A customized biocompatible polycarbrolactone O-ring (Ultimaker B.V, Netherlands) then glued onto the bottom of the transwell using bio-compatible adhesive (Kwik-cast sealant, world precision instrument, Sarasota, FL, USA). The O-ring was used to prevent any leakage from the hydrogel. The transwell apical side then treated with 50μL fibronectin (0.03 mg/mL, Thermo Fisher Scientific, Waltham, MA, USA) for 1 hr at room temperature followed by aspirating and air-drying overnight. The basal side of the assembled transwell was treated with oxygen plasma for 30 minutes before bioprinting, for activating the cell adherent surface and sterilization purposes. The final culture area was $0.143cm^2$ for 96 well HTS plate.

To perform bioprinting of 3D human dermal equivalent, a mixture of fibrinogen (2.5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA), Novogel component 2 (3 mg/mL, Organovo, Sand Diego, CA, USA) and aprotinin (0.075 U/mL, Sigma-Aldrich, St. Louis, MO, USA) was developed as printing hydrogel. The

printing hydrogel was kept in a 37°C water bath for 1 hour before use. $8x10^6$ neonatal fibroblasts were suspended with 1 mL of the warm liquid hydrogel in a 2.5 mL syringe (regenHU, Switzerland). The cell suspension was kept in the fridge for 10 minutes to allow gelation before assembled on to the bioprinter. The predefined construct was fabricated using RegenHU 3D Discovery™ Evolution bioprinter with an extrusion-based printing head. There were 3 layers of stripes printed for each tissue. 5 µL bioink was printed with U shape patterns onto 96 well HTS transwell which was subsequently filled with 25 µL fibrinogen (4.5 mg/mL), aprotinin (0.075 U/mL) and thrombin mixture (0.01 U/mL). Medium was added after 15 minutes to allow partial fibrinogen polymerization. Serum free medium (SFM-A) was mixed with 1U/mL thrombin for 24 hours (*79*). The tissue was kept at room temperature for 2 hours for further polymerization and moved to the incubator subsequently. The dermal layer was first incubated for 7 days with SFM-A (*79*). The medium was changed every other day.

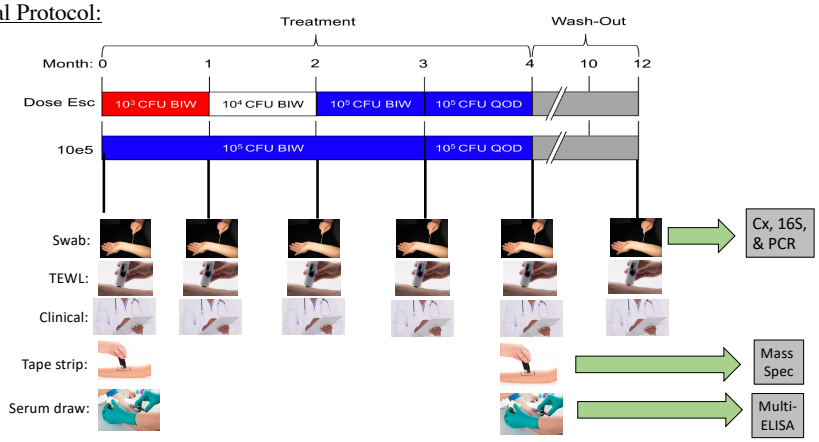
For generation of full-thickness skin equivalent (FTS), the development of full-thickness human FTS includes three phases: generating the dermal equivalent, submerged co-culture of dermis and keratinocytes, and keeping the construct at the air–liquid interface (ALI). On day 7 after bioprinting, keratinocytes (2.0×10^5 /cm^2) were seeded on the dermis. The keratinocytes were suspended with 50 µL serum-free KGM medium. The tissue was incubated with 50 µL medium in the apical side and with 400 µL medium in the basal side of the transwell. After 48 hrs, the apical side media was changed to differentiation medium, CnT-PR-3D (Zen-Bio, Research Triangle Park, NC, USA) medium supplemented with DMEM (60:40), for another 24 hours (*78*). A customized fitted lifter was added to the transwell in order to create enough medium volume in the basal compartment. The basal medium was SFM-B during this period. After 3 days, the tissue was cultured at ALI for 12 days with 350 µL SFM-C in the basal compartment.

After 10 days ALI culture, *R.mucosa* was applied directly to the surface of the tissue. Frozen stock was first thawed and resuspended with PBS. A 5 µL solution contains $2 x10^4$ cells was applied to the 0.143 cm^2 tissue surface and incubated for 2 days.

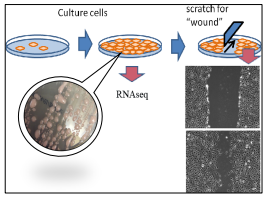
TEER measurements were acquired after 2 days *R.mucosa* culture using an automated TEER measurement system (world precision instrument, Sarasota, FL, USA). The transwell was filled with 100 µL dPBS in the apical region. TEER final values in Ω*cm^2 are obtained by multiplying the electrical resistance with skin surface area. Any tissue with a TEER value lower than 500 Ω*cm^2 was not used in this research.

Supplementary Figures:

Clinical Protocol:

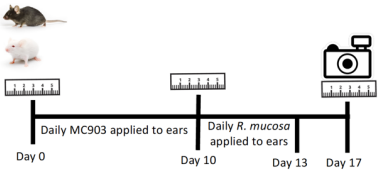


Cell Culture Protocol:



Conditions Used	
Bacterial stimulants:	*Rm*HV *Rm*AD *S. aureus* AD CoNS HV Lipid fraction *Rm*HV Aqueous fraction *Rm*HV
Antibodies:	TNFR1 TNFR2 IL8R (CXCR2) TGFb cMet TLR2 TLR4 TLR5
Modifiers to bacterial stimulants:	Lipase Atropine Mecamylamine Ketanserin
Synthetic compounds:	Flagellin Carbachol

Mouse Model:



Conditions Used	
Mouse Strains:	Wild Type (Balb) CXCR2ko (Balb bkgrd) Wild Type (C57BL/6) TNFR1ko TNFR2ko TNFRdko nAChRa7ko TLR2ko C3H TLR5ko mutStat3 NFkBko MyD88ko
Diluents for RmHV:	Sucrose (control) Lipase Atropine Mecamylamine Ketanserin

Bacterial Comparisons:

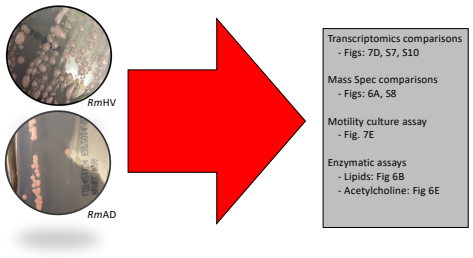


Fig. S1. Schematic indicating clinical, cell culture, and mouse protocols used in this study. Clinical protocol summary depicting Week 0-16 as well as washout visit structure. Clinical assessment involved EASI, SCORAD, CDLQI, FDLQI, and steroid use enumeration. TEWL: Trans-epidermal water loss; Cx: Culture; PCR: polymerase

chain reaction: 16S: microbiome assessment for bacterial species. Set up for cell culture assays including scratch assay conditions (HV; healthy volunteer; AD: atopic dermatitis). Protocol and conditions for MC903 mouse model experiments; ruler indicates measurement of ear thickness. Comparisons performed on *Rm*HV versus *Rm*AD as well as figures that contain the differences found.

Supplemental Figure 2

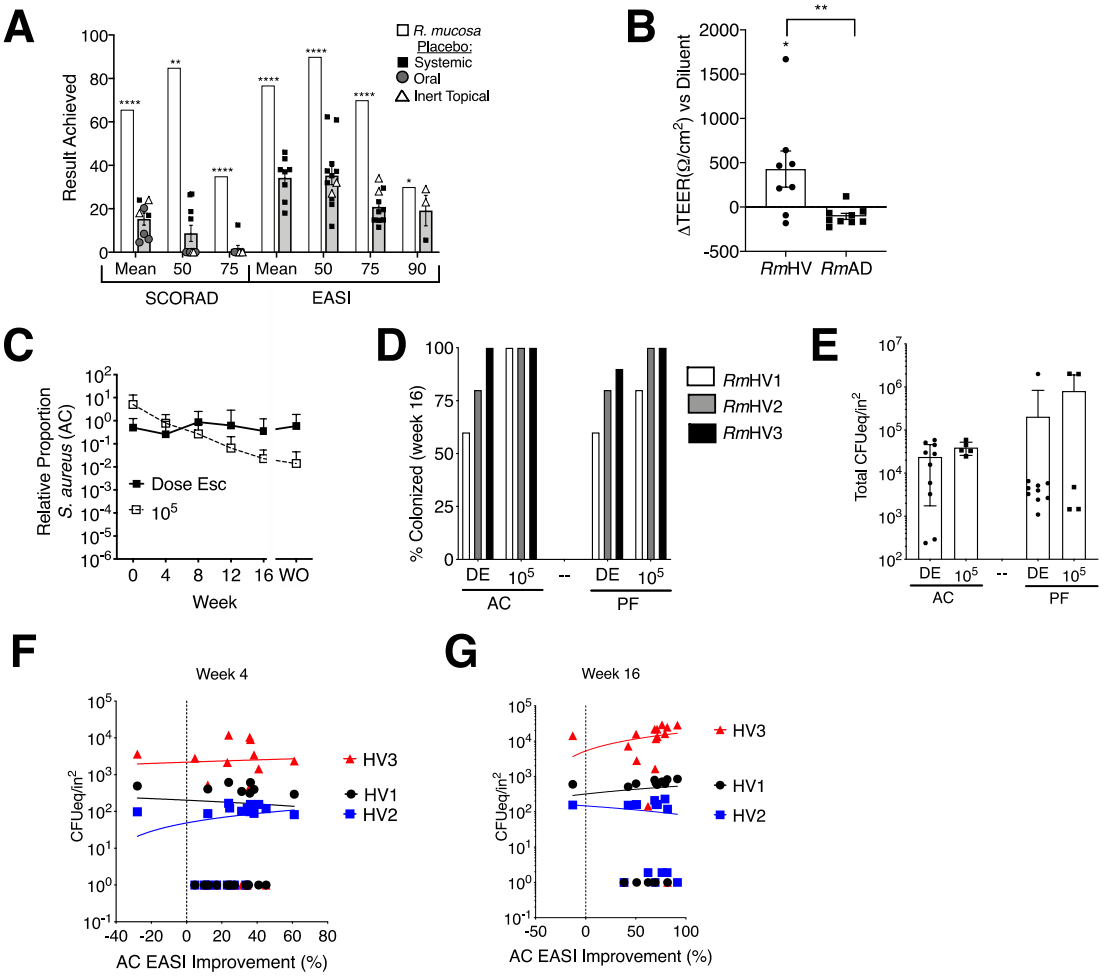


Fig. S2. *R. mucosa* treatment compared to historical placebo controls.
(A) Results for mean improvement and rates of targeted improvement for SCORAD and EASI versus placebo arms of meta-analysis from citations (*16-19*). Significant difference from the expected outcomes as calculated by Chi squared. (B) 3D printed full thickness skin cultures were stimulated with *Rm*HV1-3 or *Rm*AD1-3 at MOI=1. Trans-epidermal electrical resistance (TEER) was measured after 2 days; difference from average diluent control was calculated and shown. Data represent two independent experiments with 3-4 replicates per plate. (C) Mean +/- SEM values for relative abundance of *S. aureus* versus coagulase-negative *Staphylococci* at antecubital fossa (AC) for all patients. (D) Percent of patients with positive PCR results by body site and (E) total *Rm*HV PCR-primer signal by body site (AC = antecubital; PF = popliteal fossae) and dosing regimen (DE = dose escalation) from week 16. (F-G) Linear correlation between antecubital EASI improvement at week 4 (F) and week 16 (G) versus qPCR signal from week 4 and week 16 swabs for each *Rm*HV treatment isolate. *P < 0.05, **P < 0.01, ****P <.0.0001 versus diluent control or as indicated.

Supplemental Figure 3

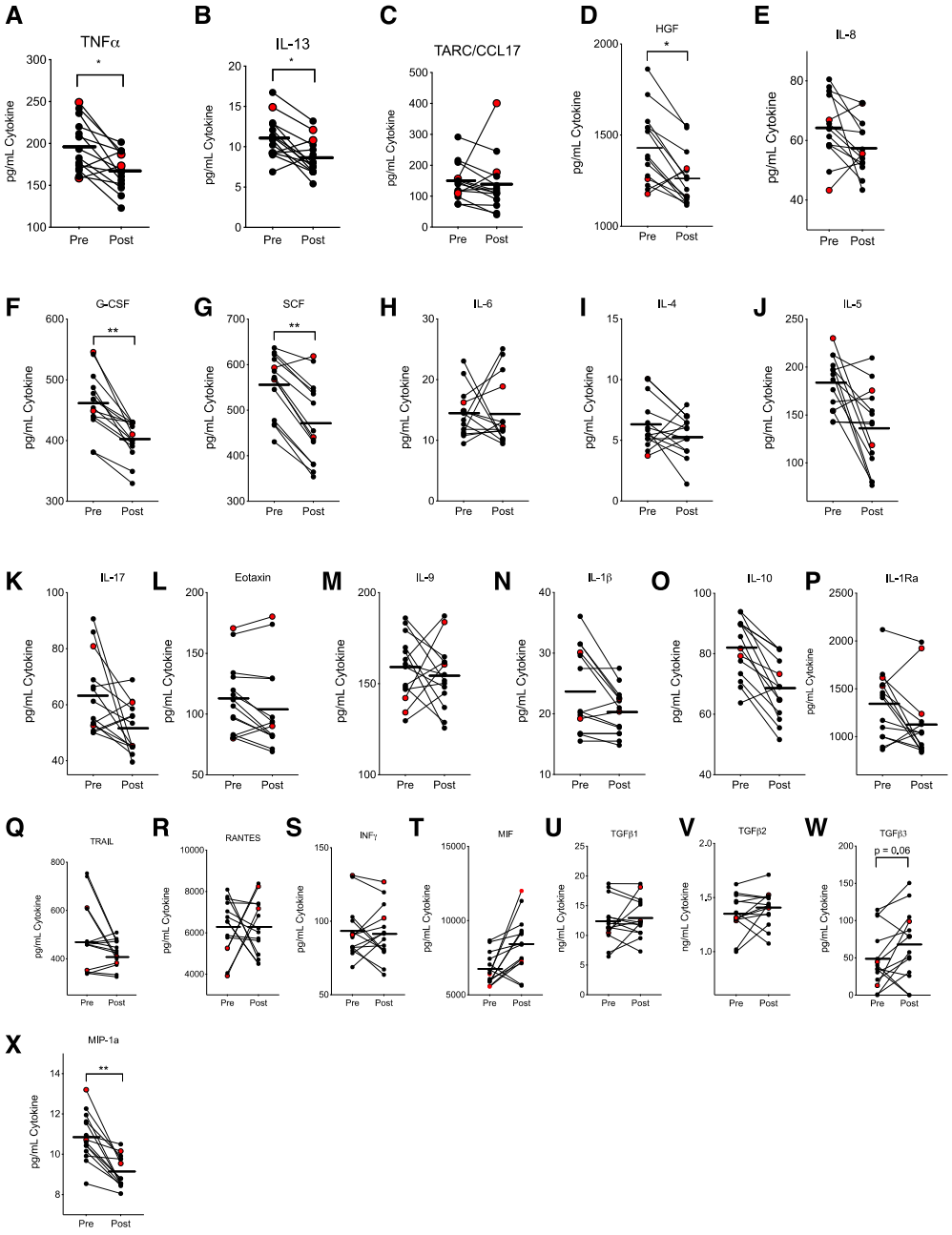


Fig. S3. Changes in serum cytokines associated with *R. mucosa* treatment. (A-X) Pre and post treatment serum concentrations for final 14 pediatric enrollees whom completed the protocol are shown. Significant differences calculated by paired t-test with adjustment for 24 comparisons by Bonferroni. Red dots indicate non-responding patients in the cohort, lines indicate mean values.

Supplemental Figure 4

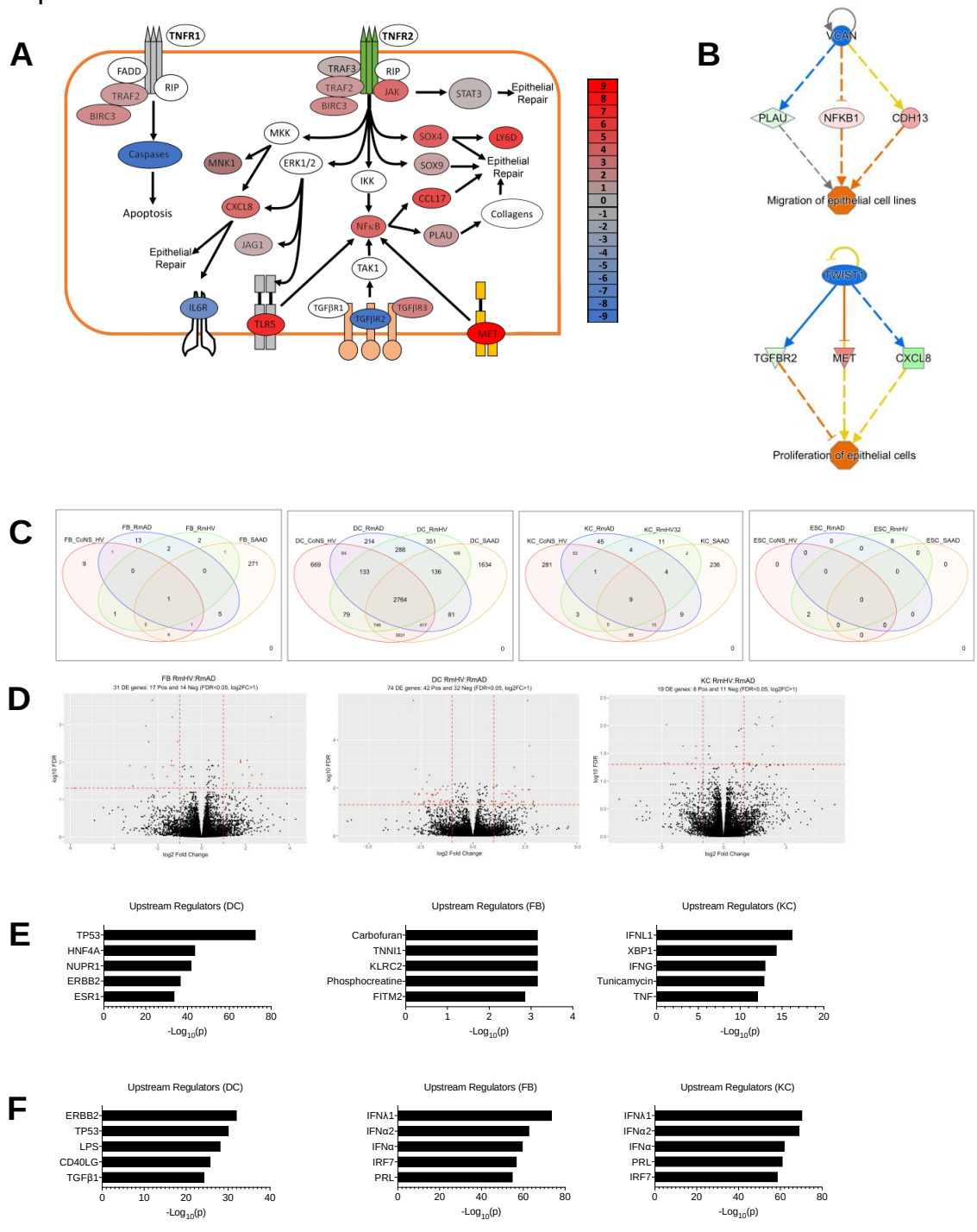


Fig. S4. *R. mucosa* from healthy volunteers activates epithelial cell migration and proliferation. (A) Summary of most altered Log_2 fold-change (Log_2 FC) RNAseq transcript abundance for indicated molecular targets and pathways indicated by Ingenuity Pathway Analysis (IPA) is presented for primary human cell cultures for keratinocytes (KC), fibroblasts (FB), dendritic cells (DC), and epithelial follicle stem cells were stimulated for 24

hours with multiplicity of infection (MOI) =1. Coloring of target indicates greater (red) or lesser (blue) Log_2 fold-change (FC) RNAseq transcript abundance averaged for all cell types stimulated with *Rm*HV versus *Rm*AD. White coloring indicates no statistically significant differences. (B) Pathway extraction directly from Ingenuity Pathway Analysis for indicated and combined cell types as assessed after *Rm*HV versus *Rm*AD stimulation. (C) Venn diagrams for transcripts that significantly differed from diluent control for fibroblasts (FB), dendritic cells (DC), keratinocytes (KC), and epithelial follicle stem cells (ESC). Cells were stimulated with *R. mucosa* from healthy volunteers (*Rm*HV), *R. mucosa* isolates from patients with atopic dermatitis (*Rm*AD), coagulase negative *Staph*. from HV (CoNS_HV), or *S. aureus* from patients with AD (*Sa*AD). (D) Volcano plots for log_2 fold-change in levels versus log_{10} adjusted p value (false discovery rate, FDR) for FB, DC, and KC comparing transcripts that uniquely differentiated stimulation with *Rm*HV from stimulation with *Rm*AD; ESC did not reveal significant differences in *Rm*HV stimulated versus *Rm*AD comparison. (E-F) Ingenuity Pathway Analysis extraction for most altered pathways for indicated cell types stimulated with CoNS stimulated condition versus diluent (E) or CoNS stimulated condition versus the *Sa*AD stimulated condition (F). Full data set uploaded under accession numbers: GSE146184.

Supplemental Figure 5

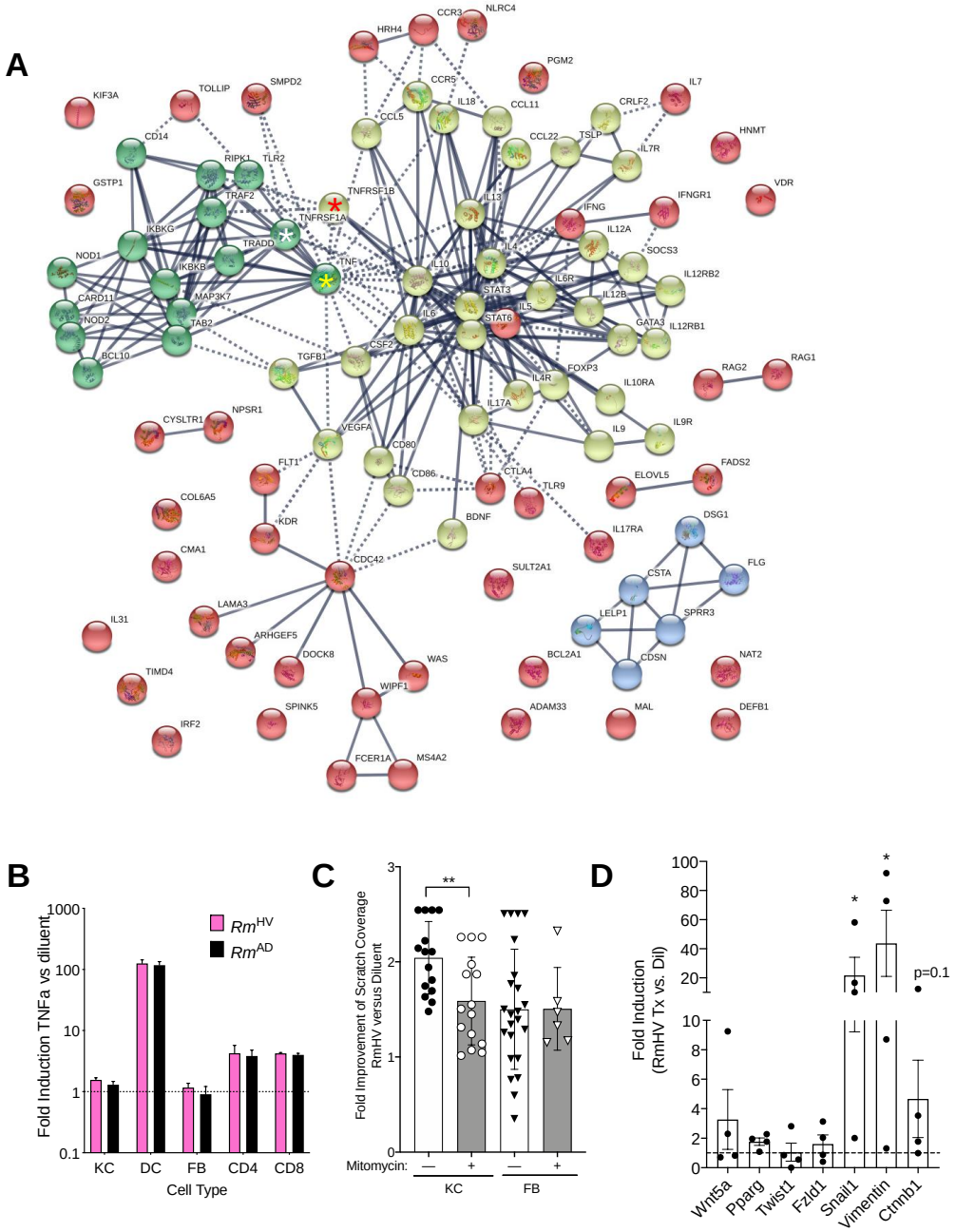


Fig. S5. TNF may be a mediator of AD. (A) Full gene list taken from (Bin et al, *Allergy Asthma Clin Immunol*, 2016) was inserted into STRING pathway analyzer. Interactions were set as "highest confidence", k means squared limit to 4 clusters, and <20 interactions per first shell. Asterisks were added to highlight the central location of TNF (yellow), TNFR1 (white) and TNFR2 (red). No further manipulations were performed. (B) Fold induction versus media control for TNFα from supernatants of keratinocytes (KC), dendritic cells (DC), fibroblasts (FB), as well as CD4+ and CD8+ T-cells. (C) Scratch assay results for KC and FB comparing experiments with and without pre-treatment with mitomycin C (and inhibitor of cellular proliferation). (D) Littermate WT mice were treated with *Rm*HV in the MC903 model as described in Fig. S1C. Transcript levels from homogenized skin harvested on day 17 relative to diluent treated WT mice (set to 1, dotted line), normalized to GAPDH, are shown as indicated. Figures B-D represent three or more independent experiments. *P<0.05, **P < 0.01 as determined by Student T test.

Supplemental Figure 6

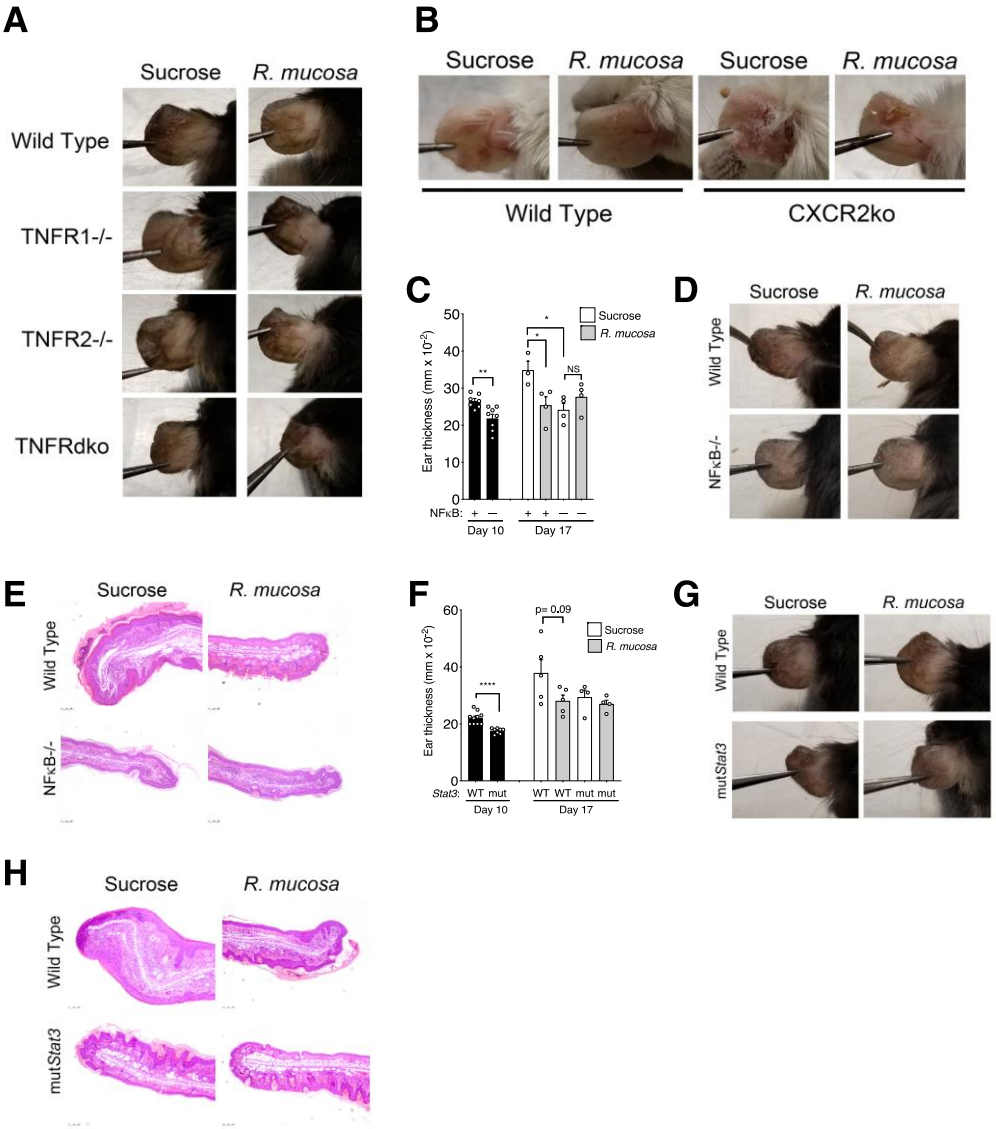


Fig. S6. *R. mucosa* activity is dependent on NFκB, STAT3, TNFR, and CXCR2. (A-B) Day 17 image for wild type (WT), TNFR1$^{-/-}$, TNFR2$^{-/-}$, and dual knock out TNFR (TNFRdko) mice (A) and CXCR2$^{-/-}$ mice (B). For WT and NFkB$^{-/-}$ mice, ear thickness (C), representative image (D) and H&E histology (E) for representative mouse in MC903 treatment with sucrose (diluent) or *Rm*HV1-3; dots in C represent individual mice. (F) Mean + SEM ear thickness after MC903 application (day 10) and after treatment (day 17) for wild type (WT) and mut*Stat3* mice. Scale bars indicate 100 micrometers. (G-H) Day 17 Image (G) and histology (H) for WT and mut*Stat3* mice. Images represent two (B-H) or three (A) independent experiments. *P < 0.05, **P < 0.01, NS = not significant as determined by T-test within groups.

Supplemental Figure 7

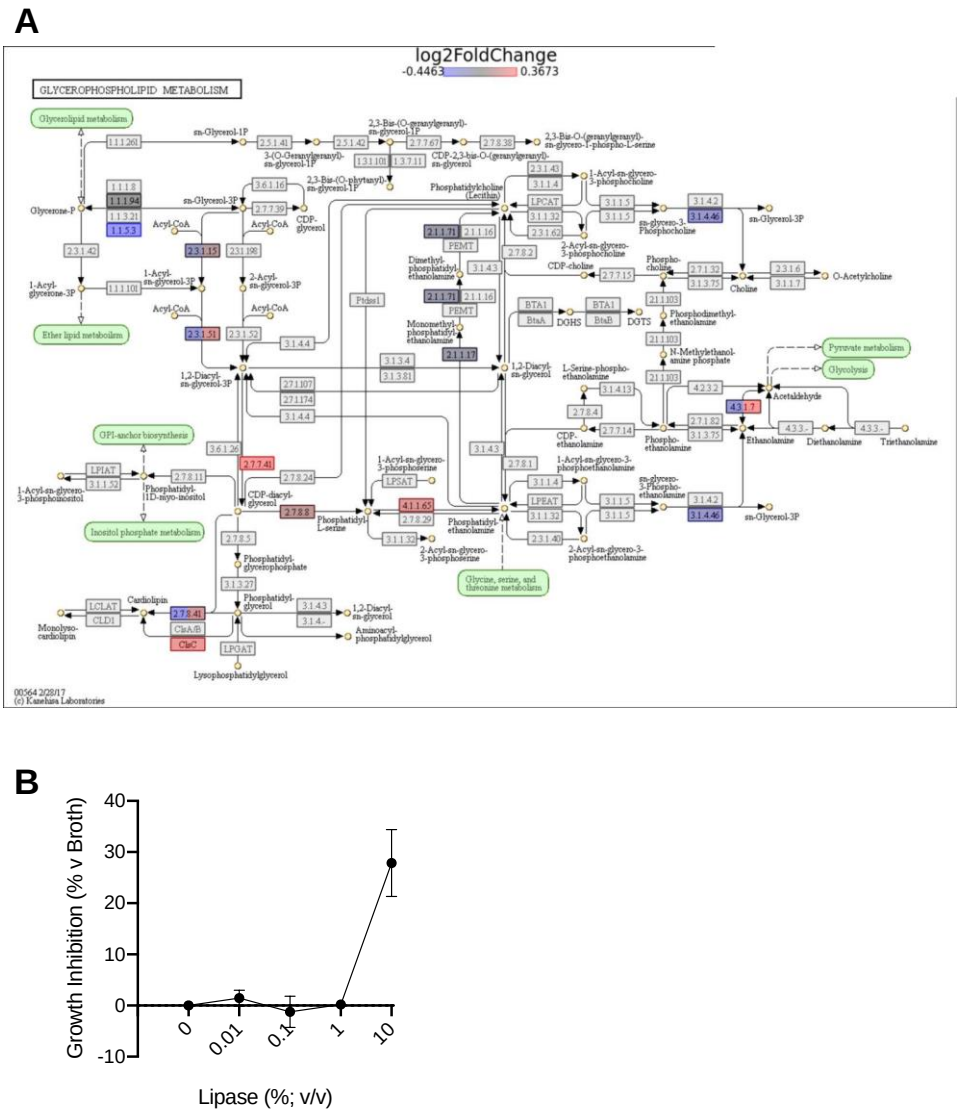
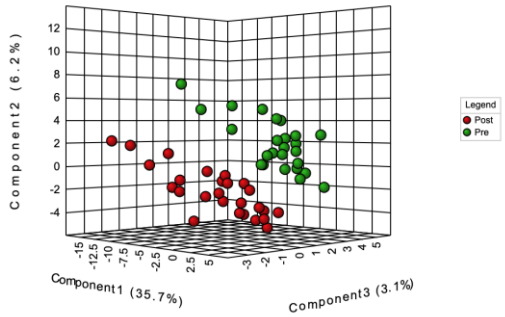


Fig. S7. *R. mucosa* from individuals with AD shows reduced phospholipid production. (A) Log_2 fold change of *Rm*HV1-3 versus *Rm*AD1-3 isolates for glycerophospholipid metabolism as determined by MEGAN KEGG pathway analysis. Red coloring indicates higher transcript abundance in HV strains, blue indicates higher abundance in *Rm*AD. (B) *R. mucosa* isolates HV1-3 were cultured with and without 1% lipase from *Candida* spp. At 24 hours absorbance for the lipase containing culture was divided by the absorbance for the broth control to derive the percent inhibition of growth; data shown is mean+SEM from the three combined isolates per experiments. Data is representative of two (A) or three (B) independent experiments.

Supplemental Figure 8

A



B

Log2 FC	-Log10(p)	Match 1	Match 2	Match 3	Match 4
3.2103	1.5651	PS 27:3; [M+Na]+			
2.8231	1.3573	SM 19:1; [M]+			
2.4408	3.5656	SM 18:1; [M]+			
2.1946	3.3384	CerP 24:1; [M+H]+			
1.7963	1.6015	PE 36:5; [M+Na]+	PC 33:5; [M+Na]+	PE 38:8; [M+H]+	
1.6211	1.5012	PC 23:2; [M+Na]+	PE 26:2; [M+Na]+	PC 25:5; [M+H]+	PE 28:5; [M+H]+
1.5068	1.3465	PS 26:2; [M+H]+			
1.4998	1.3326	PA 28:1; [M+Na]+			
1.4541	2.0185	plasmenyl-PE 27:0; [M+Na]+	plasmenyl-PC 24:0; [M+Na]+	lysoPC 24:1; [M+Na]+	
1.3405	2.4124	PC 45:6; [M+H]+	PE 48:6; [M+H]+		
1.1848	1.8202	PS 27:4; [M+Na]+			
1.0346	1.9955	PA 42:4; [M+Na2-H]+	PA 44:7; [M+Na]+		
-1.0959	2.264	PE 6:0; [M+H]+			
-1.1253	1.7445	PA 8:0; [M+Na2-H]+			
-1.1621	2.299	MG 8:0; [M+NH4]+			
-1.2688	2.2295	MG 3:0; [M+NH4]+			
-1.2928	1.4803	CerP 21:0; [M+H]+	lysoPE 16:0; [M+H]+		
-1.3087	1.5464	PE 4:0; [M+H]+			
-1.4436	4.1129	PC 14:0; [M+Na]+	PE 17:0; [M+Na]+		
-1.4949	1.3683	CerP 21:0; [M+H]+	lysoPE 16:0; [M+H]+		
-1.5759	2.2424	CerP 19:1; [M+H]+			
-1.6968	2.0642	PE 5:0; [M+H]+			
-1.9538	1.5106	lysoPC 2:0; [M+H]+	lysoPE 5:0; [M+H]+		
-2.079	1.3886	PA 19:1; [M+Na2-H]+	PA 21:4; [M+Na]+		
-2.0984	2.2639	PA 17:0; [M+Na]+			
-2.1217	1.8838	PC 5:0; [M+H]+	PE 8:0; [M+H]+		
-2.2274	4.0608	MG 6:0; [M+NH4]+			
-2.5006	2.4455	CerP 38:2; [M+H]+			

Fig. S8. Metabolomics assessment of skin tape strips indicates lipid pathway modulation. (A) Partial least squared regression comparison by MetaboAnalyst (A) and Log_2 fold change of lipids (B) reliably annotated in the pre- versus post-treatment tape strip samples as derived by LIPID Maps (N = 13; two layers of strips per).

Supplemental Figure 9

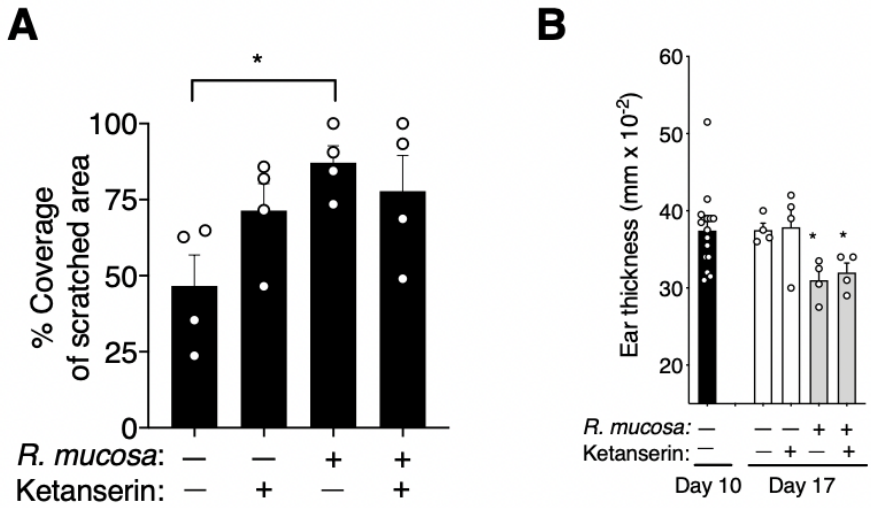


Fig. S9. *R. mucosa* activity was not influenced by serotonin receptor blockade. (A) Coverage area for fibroblasts treated with *Rm*HV1-2 with and without 10nM ketanserin. (B) Mean ear thickness after MC903 application (day 10) and after treatment (day 17) for wild type treated with *Rm*HV1-2 suspended in either sucrose or sucrose with 100nM ketanserin. *P < 0.05 as calculated by ANOVA.

Supplemental Figure 10

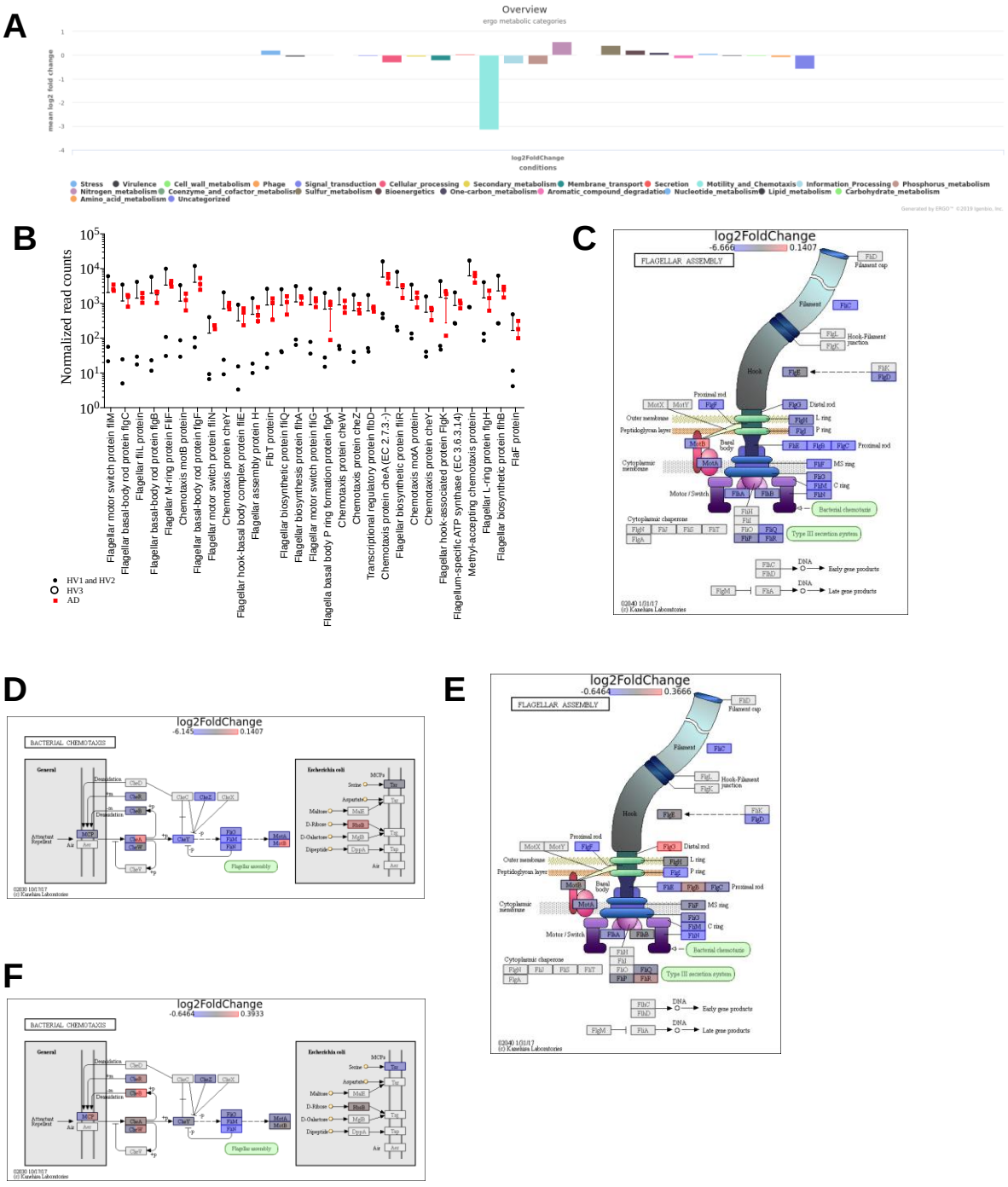


Fig. S10. *R. mucosa* from individuals with AD show increased chemotaxis and flagellin transcript production. Differential gene expression (DGE) analysis was performed on three HV

and three AD sourced *R. mucosa* isolates. (A) Ergo pathway overview for global DGE analysis for *Rm*HV1-2 versus *Rm*AD1-3. Flagellar and chemotaxis pathways were significantly different for *Rm*HV1-2 versus *Rm*AD1-3; no pathways were significant for *Rm*HV1-3 versus *Rm*AD1-3. (B) Normalized read counts for indicated genes for each isolate as indicated. (C-D) Expression overview for flagellar construction (C) and chemotaxis (D) for *Rm*HV1-2 versus *Rm*AD1-3. Red coloring indicates higher expression in HV strains, blue indicates expression in *Rm*AD. (E-F) Expression overview for flagellar construction (E) and chemotaxis (F) for all *Rm*HV isolates versus all *Rm*AD isolates. Red coloring indicates higher expression in HV strains, blue indicates expression in *Rm*AD. Data are representative of two independent experiments.

Supplemental Figure 11

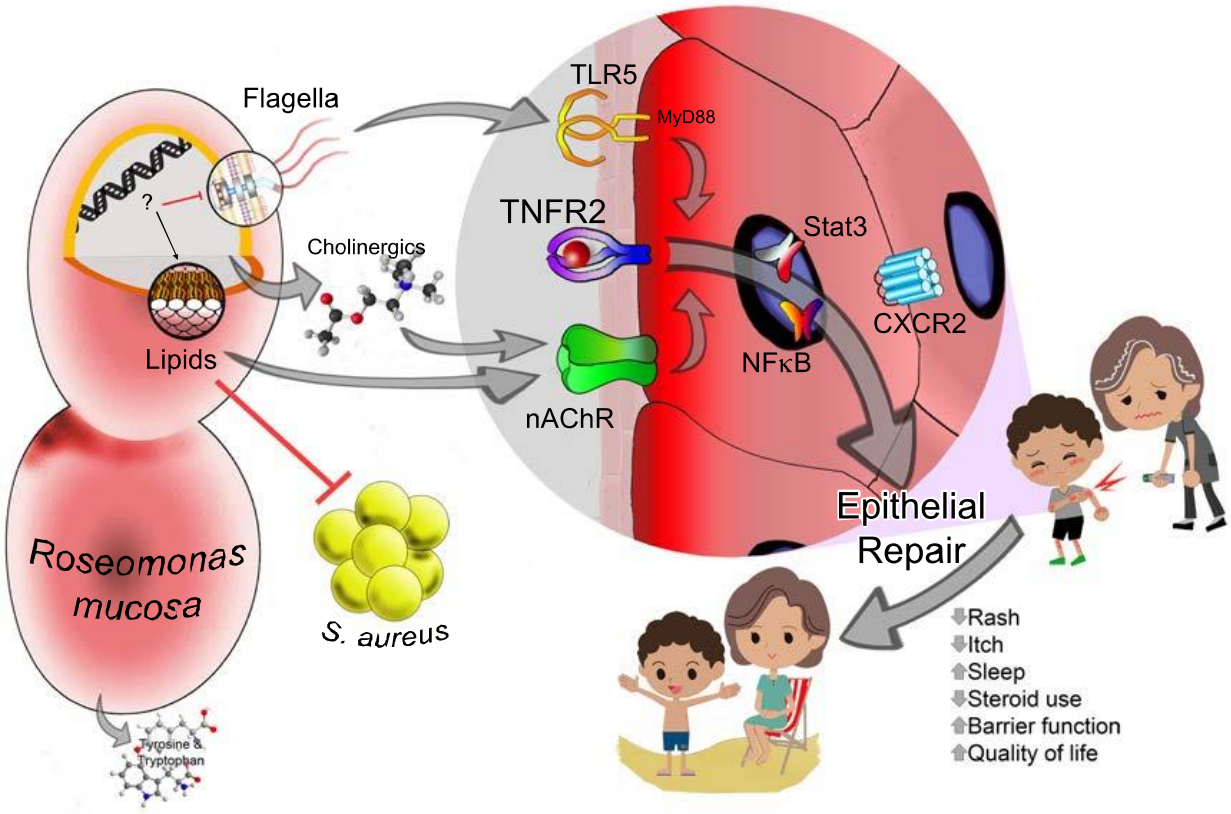


Fig. S11. Schematic showing proposed mechanisms. Unknown genetic and/or epigenetic changes lead to alterations in *Roseomonas* expression of flagellin and chemotaxis pathways alter baseline stimulation of host TLR5. In addition, health associated *Roseomonas mucosa* produces cholinergic compounds as well as unique glycerophospholipid profiles which act upon the nicotinic acetylcholine receptor (nAChR). TLR5 and nAChR both impact signaling through TNFR2, which subsequently alters downstream epithelial migration and proliferation. This process also includes CXCR2 and leads to epithelial regeneration via epithelial-to-mesenchymal transition. Lipid inhibition of *S. aureus* was demonstrated in prior publication (Myles, et al. 2018 *JCI Insight*). Although not impactful in our models, bacterial and patient metabolomic evaluations demonstrated treatment-associated difference in tyrosine and tryptophan pathways; raising the possibility of catecholamine and/or serotonergic contributions. Clinically, these modeled impacts predicted clinical improvements in rash, itch, steroid use, and quality of life.

Table S1. Demographics and baseline characteristics of patient cohort.

	Part 1 (Adult Cohort) N=10	**Part 2 (Pediatric Cohort) N=20§**
Age (years)		
Mean (min, max)	41.5 (19–70)	9.0 (3–16)
Gender, n (%)		
Male	1 (10.0%)	10 (50%)
Female	9 (90.0%)	10 (50%)
Race, n (%)		
Asian	3 (30.0%)	4 (25%)
Black	0	3 (15%)
White	7 (70.0%)	13 (65%)
Pre-enrollment treatment regimen		
Topical Steroids, OTC§	8 (80%)	9 (45%)
Topical Steroids, Prescription§	1 (10%)	8 (40%)
Calcineurin inhibitors	1 (10%)	2 (10%)
Antihistamines	2 (20%)	13 (65%)
Mupirocin	0	4 (20%)
Coconut oil	0	2 (10%)
Phototherapy	0	1 (5%)
Crisaborole	0	3[#] (15%)
Dupixent	0	1* (5%)
Mean (Min, Max)	**Part 1 (Adult Cohort) N=10**	**Part 2 (Pediatric Cohort) N=20§**
SCORAD	11.7 (4–17) (antecubital SCORAD)	36.1 (12.8–73.2) (total body SCORAD)
Pruritus score (0–10 scale)	4.6 (2–10)	6.6 (3–9)
EASI	—	7.8 (1.6–23.3)
BSA affected by AD	—	6.1 (2.1–13.6)
FDLQI	—	12.2 (3–23)

§ = analysis limited to patients that completed treatment.

* = discontinued prior to enrollment due to reported lack of efficacy.

= Two discontinued prior to enrollment, one discontinued at week 6 due to reported side effects and lack of efficacy.

§ = All topical steroid usage was reported as stable for months to years prior to enrollment for all patients. Week 0 steroid usage reflects average steroid use over the four months prior to enrollment.

Table S2. Clinical characteristics of participants enrolled in the study.

Patient	Age	Sex	Week 0 IgE	Week 16 IgE	Asthma	Allergic rhinitis	Food allergy	Eosinophilia	Contact derm	Drug allergy	Percent improvement SCORAD	Percent improvement EASI
1	9	F	26.2	17.1							41.9	62.5
2	14	M	381	375			X				53.1	71.2
3	10	F	1163	1256	X	X	X				100	100
4	10	F	4.5	4.7					X		100	100
5	9	F	6920	4233	X	X	X	X			60.3	78.7
6	14	M	383	313							69.3	93.6
7	5	F	2620	3379		X		X			50,9	52.4
8	4	M	489	508	X	X	X				81.8	92.6
9	5	F	1975	1958	X		X	X		X	42.4	6.1
10	14	F	1809	1224		X	X				62.3	80.3
11	3	M	789	676			X				81.4	95.8
12	3	M	12.2	10.7		X	X				91.6	86.1
13	10	F	755	1102			X				71.4	93.6
14	12	F	5891	6543	X	X	X		X		68.8	88.6
15	15	M	2175	2070							-12.9	32.3
16	4	M	390	443		X	X				71.2	76.7
17	14	M	448	647	X	X	X				79.2	88
18	3	F	58.5	66.1			X		X		50.3	76.1
19	12	M	6335	4902		X	X			X	70.1	72.5
20	16	M	1294	1715	X	X	X				76.2	89.1

Table S3. Clinical improvement after *R. mucosa* treatment stratified according to EASI severity at enrollment.

EASI Measures	Mild Disease EASI ≤ 7 at Baseline N=11	Moderate/Severe Disease EASI > 7 at Baseline N=9
EASI50	9 (81.8%)	9 (100%)
EASI75	6 (54.5%)	8 (88.9%)
EASI90	3 (27.3%)	3 (33.3%)

Table S4. Treatment emergent and adverse events after *R. mucosa* treatment.

	Adult cohort (n=10)	Pediatric cohort (n=21)
Treatment-related adverse reactions*, n (%)		
Application site pruritus	0	1 (5)
Treatment-related adverse events§, n (%)		
Application site pruritus	0	0
Application site pain	0	0
Fever	0	0
Discoloration	0	0
Worsening pruritus	0	0
Worsening SCORAD	0	0
Infection, skin	0	0
Infection, other	0	0
Injury	0	0
Headache	0	0
Cough	0	0
Lab abnormalities (see methods)	0	0
Unrelated adverse events#, n (%)		
Viral upper respiratory infection	0	2 (9.5)
Non-anaphylactic reaction to known food allergens	0	3 (14)
Hand foot and mouth disease during regional outbreak	0	1 (5)

*Problems attributable to therapy by timing; self-limited and did not interfere with patient's daily activities or treatment compliance.
§Problems related to therapy by timing; persistent and/or interfering with patient's daily activities or treatment compliance.
#Problems related to therapy by timing but not causal relationship to treatment.

Table S5. Primers used for strain-specific identification.

Roseomonas strain	GenBank	Oligo Name	Type of oligo	5'-3' sequence	Amplicon (bp)
HV2	RSQQ03345	169RSQQ03345F	For	GGCGGGTCTGTTGCACTT	61
	RSQQ03345	239RSQQ03345R	Rev	GGCTCCGATGCCGTCTT	65
	RSQQ03345	191RSQQ03345CFO	CFO-BHQ1	CGCGGCCCAAACAGGCCA	72
HV3	RKNM01394	592RKNM01394F	For	GCGGCAAGCATTCACCTT	56
	RKNM01394	700RKNM01394R	Rev	GCTGATCGCCTTGACGTAACTC	55
	RKNM01394	624RKNM01394FA	FAM-BHQ1	TGGCGATGCTCCTCGGCTTCA	62
HV1	RHFA01191	721RHFA01191F	For	CCCAAGATTGCAGCCAGTCT	55
	RHFA01191	827RHFA01191R	Rev	TTAAGGACGGTCACATTCAGCTT	43
	RHFA01191	770RHFA01191Q6	Quasar670-BHQ2	CGAACAGTGCTGTCCGCATTGCA	57